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Preservation of Infective Stages of Rodent Malaria

by

C. P. A. Strome

and

J. L. Leef

Biomedical Research Institute
12111 Parklawn Drive
Rockville, Md. 20852

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A major portion of our efforts involved training personnel, setting up and maintaining insectaries and animal facilities, establishing the cyclical transmission of malarial parasites and fully equipping our cryobiological laboratory. Once routine transmission was established and techniques for isolating sporozoites for experimental manipulation were worked out, sporozoite

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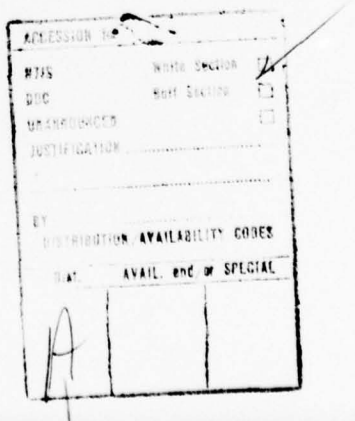
freezing experiments were initiated. A series of base-line experiments to determine the effect of the preservative alone, Dimethyl Sulfoxide (DMSO), on Plasmodium berghei sporozoites before freezing proved uninformative because the infectivity of the parasites was very low even at the highest dose level used. During this time the temperature and humidity in the insectaries had been fluctuating erratically, so the freezing experiments were stopped until that situation could be corrected and satisfactory levels of infectivity could be attained. Until the environmental conditions were stabilized, infectivity studies showed a continuous decrease in sporozoite infectivity. After proper repairs were made, the erratic temperature and humidity fluctuations ceased and the infectivity levels gradually returned to satisfactory. The freezing studies were resumed; however, we found that most sporozoites merely exposed to DMSO were non-infective before the freeze-thaw event. A new source of DMSO was found which had previously been shown to be non-toxic to tissue culture cells and sporozoites were exposed to various concentrations of this DMSO. The preliminary results indicated that exposure to the new DMSO had no effect on the infectivity of the sporozoites. Repetition of this experiment along with freezing sporozoites in the new DMSO are currently in progress.

Abstract

A major portion of our efforts involved training personnel, setting up and maintaining insectaries and animal facilities, establishing the cyclical transmission of malarial parasites and fully equipping our cryobiological laboratory. Once routine transmission was established and techniques for isolating sporozoites for experimental manipulation were worked out, sporozoite freezing experiments were initiated. A series of baseline experiments to determine the effect of the preservative alone, Dimethyl Sulfoxide (DMSO), on Plasmodium berghei sporozoites before freezing proved uninformative because the infectivity of the parasites was very low even at the highest dose level used. During this time the temperature and humidity in the insectaries had been fluctuating erratically, so the freezing experiments were stopped until that situation could be corrected and satisfactory levels of infectivity could be attained. Until the environmental conditions were stabilized, infectivity studies showed a continuous decrease in sporozoite infectivity. After proper repairs were made, the erratic temperature and humidity fluctuations ceased and the infectivity levels gradually returned to satisfactory. The freezing studies were resumed; however, we found that most sporozoites merely exposed to DMSO were non-infective before the freeze-thaw event. A new source of DMSO was found which had previously been shown to be non-toxic to tissue culture

cells and sporozoites were exposed to various concentrations of this DMSO. The preliminary results indicated that exposure to the new DMSO had no effect on the infectivity of the sporozoites. Repetition of this experiment along with freezing sporozoites in the new DMSO are currently in progress.



The following report describes our progress in developing a procedure leading to the long term preservation of sporozoites of Plasmodium berghei at low temperature. The training of personnel, setting up and maintaining mosquito colonies, insectaries and animal facilities and cyclical transmission of the malaria parasite was the preoccupation of the first half of the contract reporting period. In addition, a detailed analysis of the "state of the art" of the cryopreservation of malaria parasites has been made from the literature and can be found in Appendix I.

Once routine transmission was established, experimental procedures were worked out for handling sporozoite material and quantifying it for use in subsequent protocols. In addition, adjustments in the environmental conditions had to be made before infectivity experiments were satisfactory. The results of fluctuations in the environment are described below and representative data are given in Tables 1 and 3. The procedures for preparing inocula currently being followed are outlined below.

Suspensions of sporozoites are routinely obtained by homogenizing whole female Anopheles stephensi mosquitoes infected 21 days earlier with P. berghei in a mortar containing normal mouse serum. The crude homogenate is diluted with medium 199 and centrifuged at 100 x g for 5 minutes to settle

the large mosquito parts. The supernatant is centrifuged at 16,000 x g for 15 minutes to sediment the sporozoites and small debris. This sediment is then resuspended in 1 ml of 199, layered onto a hypaque density gradient containing hypaque, medium 199 and mouse serum. After centrifugation for 30 minutes at 16,000 x g, the area richest in sporozoites is visible and is collected by drawing it off with a syringe and needle. The material is then diluted with medium 199 and centrifuged at 16,000 x g for 25 minutes, resuspended in 1 ml of 199 and the number of sporozoites estimated by hemocytometer count. The material, which is kept at 0°C throughout this procedure, is then ready for experimental manipulation. Following a given experiment, female mice, NIH/NMRI strain, at least 6 weeks old are injected intravenously via the tail vein with a determined number of sporozoites contained in a volume of 0.1 ml. Blood smears are made on day 7, 9 and 16 post inoculum, stained with Giemsa's stain and the precent parasitemia is determined.

Freezing studies were initiated with a series of baseline experiments to determine whether or not the preservative of choice, dimethyl sulfoxide (DMSO), had any adverse effect on the infectivity of sporozoites of Plasmodium

berghei. Sporozoites were exposed to concentrations of either 0, 5, 10 or 15% DMSO both at 0°C and room temperature for 30 minutes before injection. Aliquots of 3,000, 15,000 or 30,000 sporozoites were injected into mice to determine infectivity as described above.

The results indicated very low infectivity of any of the sporozoite suspensions; only 9 animals of a total of 120 became infected. Furthermore, no useful information regarding any effect of DMSO was obtained because infected animals were randomly scattered throughout the groups with no pattern discernible. The number of mice inoculated with sporozoites exposed to 15% DMSO that became infected equaled the number becoming infected by sporozoites exposed to 5% DMSO; mice given sporozoites exposed to 10% DMSO developed no infections at all. However, during this period of time, despite repeated consultation with the manufacturer, we were experiencing erratic temperature and humidity fluctuations in our insectaries and further experimentation with sporozoites was postponed until satisfactory and reproducible levels of infection could be attained with untreated sporozoites. Therefore, a series of mice were injected with normal untreated sporozoites to establish such a baseline. The results of these experiments are summarized in chronological order in TABLE 1. At a dose of 40,000 sporozoites per injection, a steadily decreasing trend in the infectivity was observed

as a function of time starting with the earliest result of 90% and ending with complete loss of infectivity. A similar trend was observed at the level of 20,000 sporozoites per injection.

From routine microscopic examination of mosquito stomachs and salivary glands, it was known that sporozoites were migrating from the stomach to the salivary glands and thus inocula could be presumed to contain mature sporozoites; yet these inocula were not infective. On the other hand, the majority of these sporozites obtained from whole body homogenizations could have been from the abdomen and thus would be largely immature noninfective sporozoites. In a series of four experiments summarized in Table 2, 50 mosquitoes per experiment were dissected and the thoraces and the abdomens ground separately and sporozoites isolated from each preparation as previously described. Hemocytometer counts were done and sporozoites from thoraces were injected into mice at a dose of 30,000 per mouse inoculum. In one case the sporozoites isolated from abdomens were also inoculated. We found that although the percentage of the total sporozoites obtained from the thorax varied considerably the mean percent of thoracic sporozoites was rather high (30%). The infectivity of thoracic sporozoites was 41% (Table 2), approximately the same as the whole body homogenates reported in TABLE 1 at the dose level of 40,000 per inoculation where the

mean percent infectivity was 36%. Thus these results support the conclusion that substantial numbers of sporozoites were migrating to the salivary glands and were, therefore, mature. On the basis of these results, the use of whole body homogenates to prepare inocula was judged adequate.

Subsequently wiring defects and improper settings were found in the control panels for the insectaries. After repairs were made, the temperature and humidity fluctuations were stabilized and the results from infectivity studies began to improve (Table 3). Table 3 contains a summary of data acquired during this period and contrasts infectivity results prior to and after correcting the deficiencies in the control panel. The gradual increase in infectivity of the sporozoites which accompanied stabilization of the environment emphasizes the importance of careful monitoring of the environmental controls in a system such as this one.

It was felt that we had now reached a point where meaningful low temperature studies could be initiated since the environmental conditions were stable. Previous experience with P. berghei sporozoites indicated that infectivity was retained following rapid freezing (shell freezing)* in DMSO in the presence of normal mouse serum. Since we plan to examine cooling rates ranging from 1° to 500°C per minute and warming rates ranging from 25° to 1000°C

*Shell freezing commonly denotes a process by which material is frozen by agitation in a dry ice-ethanol slurry at -79°C.

per minute, we elected to start with that combination of rates which had already yielded viable parasites. Sporozoites were collected as described above and 1 ml samples were frozen at 500°C per minute (shell freezing) until reaching equilibrium with the bath (-79°C) as measured by a copper-constantin thermocouple immersed in the sample. The temperature and rate of change was recorded on a Honeywell Electronik 112 temperature recorder. After temperature equilibration, samples were transferred to liquid nitrogen for 30 minutes of equilibration at -196°C. They were thawed by immersion and rapid agitation in a 37°C water bath (warming rate 750 - 1000°C per minute) and inoculated into mice as previously described. The data from this series of experiments, summarized in Table 4, indicated three things: (1) the infectivity of untreated sporozoites is high at doses of 40,000 and 20,000 per inoculum; (2) the infectivity of unfrozen sporozoites suspended in 10% DMSO in 45% mouse serum was severely reduced compared to the untreated controls mentioned above; (3) no infectivity was observed following freezing. However, there was little chance for freezing to be successful since most of the sporozoites were rendered noninfective by exposure to DMSO alone. Toxicity

of DMSO is not a rare event and has been reported often in the literature. Therefore, we obtained a new source of DMSO which had been shown to be nontoxic to tissue culture cells. The infectivity of sporozoites was determined after being held in the new DMSO at concentrations of either 2.5, 5, 7.5, or 10% respectively for 30 minutes at 0°C; this was the same length of time the previous sporozoites were held in 10% DMSO prior to injection. Examination of Table 5 reveals no apparent deleterious effects of the new DMSO on the infectivity of sporozoites at concentrations as high as 10% when compared to sporozoites that were not in contact with DMSO; however, this must be repeated before firm conclusions can be reached. In addition, freezing experiments using the new DMSO are currently in progress.

During this time, we have also completely equipped our cryobiological laboratory and have helped complete a long term study which resulted in the two publications listed below.

1) Beaudoin, R.L., Tubergen, T.A., Strome, C.P.A., and Leef, J.L.
A Quantitative Study of the Cryopreservation of Malaria Parasites. Proc. V International Congress of Protozoology. In press. (1977).

2) Strome, C.P.A., Tubergen, T.A., Leef, J.L., and Beaudoin, R.L.
Long-Term Cryobiological Study of Malaria Parasites. Bull. W.H.O. In press. (1977).

This investigation was supported in part by the Bureau of Medicine and Surgery and the Office of Naval Research Contract N00014-76-C-0724.

TABLE 1. Number of mice infected with P. berghei sporozoites following separation by the density gradient technique. Temperature fluctuations in the insectary had not been corrected throughout the duration of these experiments.

	SPOROZOITE DOSE		
Experiment	40,000	20,000	10,000
1*	9/10**	3/10	0/10
2	5/10	2/10	0/10
3	3/10	0/10	0/10
4	1/10	0/10	--
5	0/10	0/10	--

* The first of a series of experiments which are presented in chronological order; several weeks separate experiment one from experiment five.

** The fraction of the total number of mice that developed blood stage parasites.

TABLE 2. The relative proportion of thoracic and abdominal sporozoites and the infectivity of thoracic versus abdominal sporozoites. Temperature fluctuations in the insectary had not been corrected throughout the duration of these experiments.

30,000 Sporozoites per inoculation		
	Thoracic	Abdominal
The percent of total sporozoites	30*	70*
The average percent infectivity	40.7*	20**

* The average of 4 experiments.

** A single experiment.

TABLE 3. Number of mice infected with P. berghei sporozoites following separation by the density gradient technique. Temperature fluctuations in the insectary had not been corrected during experiment 1; repairs were made while experiment 2 was in progress; repairs had been made before experiment 3 was started.

	SPOROZOITE DOSE			
Experiment	40,000	20,000	10,000	5,000
1*	0/3**	0/3	0/3	0/3
2	1/3	0/3	0/3	0/3
3	5/10	8/10	8/10	2/10

* Several weeks separate experiment one from experiment three.

** The fraction of the total number of mice that developed blood stage parasites.

TABLE 4. The percent of mice infected with sporozoites following the indicated treatments. Temperature fluctuations in the insectary had been corrected before these experiments were started.

Treatment	Sporozoite Dose		
	40,000	20,000	10,000
Untreated sporozoites	84%*	62%*	50%***
Sporozoites exposed to DMSO	15%**	25%**	--
Frozen sporozoites	0%**	0%**	--

* The mean of 5 experiments.

** The mean of 2 experiments.

*** A single experiment.

TABLE 5. Number of mice infected with P. berghei sporozoites following exposure to the indicated concentrations of "new" DMSO for 30 minutes at 0°C prior to injection. Temperature fluctuations in the insectary had been corrected before this experiment was started.

SPOROZOITE DOSE	
Concentration (%) of DMSO	45,000
0	8/8*
2.5	6/7
5.0	5/7
7.5	7/7
10.0	5/7

* The fraction of the total number of mice that developed blood stage parasites.

Appendix I

Subject - A review of the relative "state of the art" of freezing malarial parasites.

Author - James L. Leef

General Statement - This review will be divided into three sections: (I) freezing of Plasmodium sporozoites; (II) freezing of Plasmodium blood stages; (III) a brief review of freezing other protozoans. The latter is included in order to gain a general idea of techniques currently in use in freezing protozoans since there have been so few cryobiological studies done with malarial parasites. In addition, most of the freezing of malarial parasites has been done when they were in the blood stages and with few exceptions, the reports offer no insight into suggesting new, or improving existent techniques. A critique follows each section.

Review - (I) Freezing of Plasmodium sporozoites - Jeffery and Rendtorff (1955) stated that, to that date, no successful attempt to freeze sporozoites had been reported. They successfully froze human malarial sporozoites and reported that 30 out of 37 human volunteers developed malaria after receiving injections of frozen-thawed sporozoites. They found that no infections were transmitted by sporozoites frozen in saline solution alone but that all sporozoite suspen-

sions frozen in plasma retained infectivity. Bafort (1968) referred to Jeffery's work as the only successful attempt to freeze sporozoites but noted that rodent malarial sporozoites had not yet been frozen with retention of viability. He observed the appearence of blood forms in hosts after injection of freeze-thawed sporozoites from only 2 out of 26 frozen samples. He referred to a study by Steighold (1966) who reported "successful dissection of sporozoites from mosquitoes" which had been stored at low temperature for 2 1/2 years, however, no viability estimates were done on these organisms. Jakstys et al (1974) compared the ultrastructure of untreated, frozen (four times) or heat inactivated Plasmodium berghei sporozoites and concluded from morphological evidence that heat inactivation resulted in a more "natural" appearing cell than did those frozen and thawed four times. No viability or infectivity studies were done. Weathersby (1976) has freeze-dried sporozoites of P. gallinaceum and recovered infective material but the report was an abstract so the data could not be analyzed.

Critique - It is unfortunate that the above authors were not concerned with a quantitative presentation of their data. Examples: dosage was not determined by sporo-

zoite number but was recorded as number of salivary glands used or as the number of mosquitoes ground in a mortar and pestle and used per dose or treatment; viability was generally indicated by the length of prepatent periods which were extremely variable in controls and frozen material; in each study only one cooling and warming rate was examined; and only one concentration of any given preservative was used. Because of such reasons as these, one author concluded that sporozoites were quite resistant to freezing, Jeffery and Rendtorff (1955); while another felt sporozoites may be sensitive to freezing (Bafort). Thus, neither the degree to which sporozoites can withstand freezing nor the best conditions for cooling and warming are known. Since we plan to use frozen sporozoites as a source of antigen it will be necessary that they remain as biologically and physically unaltered as possible. Experiments must be designed to establish parameters for learning the optimal cooling and warming rates, and the proper concentration of given preservatives to yield maximal viability of frozen-thawed sporozoites.

Review - (II) Blood stages - Most of the low temperature studies done with malarial parasites have been with the organisms when they were in the erythrocytic stages.

The rationale was that banking infected cells at low temperature would eliminate frequent transfer in laboratory animals, a process which is not only time consuming and costly but is also plagued with loss of virulence and genetic alteration resulting in lowered antigenicity. Wolfson (1945) showed that P. cathemerium in citrated whole blood remained infective after "rapid freezing" (no rate given) but that the degree of parasitemia was only 50% of control after the initial passage. In subsequent passages it reached the same level as control. Jeffery (1957), working with P. falciparum, found that the organisms remained infective after 3 years at -79° C. Schneider and Shefner (1970) reported no decline in infectivity of P. berghei after 3 years in liquid nitrogen. Jeffery (1962) introduced the use of DMSO and glycerol in the preservation of P. berghei and P. gallinaceum and Booden and Gieman (1973) preserved P. falciparum successfully at low temperature using DMSO. The recovery was based on infectivity of frozen blood in mammalian hosts and although he reported better recovery when preservatives were used, the difference was not large. Schneider and Seal (1973) studied the influence of various cooling and warming rates on P. berghei. In

that study they presented a review of current cryobiological theory as it applied to blood parasite freezing; they varied the freezing parameters appropriately by holding the warming rate constant while varying the cooling rate and vice versa over two orders of magnitude. Their preservative of choice, when used, was 10% glycerol. After thawing they monitored the degree of parasitemia after 3, 6 and 30 days respectively in their host mice and recorded mortality. They expressed the viability and/or biological activity in terms of potency, where potency was determined by titration of a given concentration of organisms and defined as the smallest dose that infected 100% of the recipient mice. Their results, however, were quite unusual; they reported a 100 fold increase in the infecting titer following freezing under the best conditions (rapid freeze with rapid thaw) as compared to unfrozen control. They had no explanation for this phenomenon. Conversely, Diggs et al (1975) found that the infectivity of P. falciparum (in monkeys) was essentially the same as unfrozen control; but they did not investigate more than one cooling and warming rate. Wilson and Farrant (1976) found that blood parasitized with P. knowlesi showed little hemolysis

when frozen rapidly but showed negligible recovery of parasites based on incorporation of 3H-leucine, while a two step freezing method (4° to -25° then to -196° C.) resulted in little hemolysis with a high rate of incorporation of the labeled amino acid.

Critique - It is difficult to criticize these papers because they were intended only to provide a means by which blood parasites could be preserved so that frequent laboratory passages could be avoided. The desire was to retain sufficient infectivity, regardless of the percent survival, so that when thawed, the material could be used to infect animals. They have accomplished this end and low temperature banking is now a common laboratory technique. However, to my knowledge, there have been no publications regarding the freezing of gametocytes for the purpose of infecting mosquitoes rather than the infection of a mammalian host via the injection of frozen-thawed blood stages. This is the only blood stage of real interest to our program.

Review - (III) Freezing of other protozoans - In preparing this review it was found that techniques employed in the low temperature preservation of parasites at

large do not differ significantly from those used in preserving malarial parasites (Diamond, 1964; Lumsden, 1972; Schneider and Seal, 1973). These reviews of the subject also illustrate the wide range of protozoans which have been successfully frozen and those which are currently banked at low temperature. Dalgliesh and Mellors (1974) used 10% DMSO to preserve Babesia bigemina in blood and employed cooling rates ranging from 1 to 800° C/min. They reported no difference in infectivity between the various rates and obtained 95-99% infectivity. However, they injected more than 1 ml of preserved blood into each recipient so that most differences in viability which may have existed could have been masked by the large inoculum. Kocan et al (1967) and Strome (1976) have shown that Leucocytozoon simondi sporozoites survive freezing equally well in glycerol or DMSO at rapid or slow rates of cooling coupled with rapid warming. Walker and Ashwood-Smith (1960), Filardi and Brener (1975) and Diffley et al (1976) have shown that trypanosomes in the presence of glycerol or DMSO survived freezing at low rates of cooling. However, Walker and Ashwood-Smith (1960) reported a loss in motility after exposure to high concentrations of glycerol at room

temperature and Diffley et al (1976) reported similar findings with 5% DMSO. Both authors interpreted loss of motility as a toxic effect of the preservative. Neal et al (1974) reported the survival of amoeba after freezing in 7.5% DMSO at 1°/min. to -30°C. and then transferred directly to liquid nitrogen. Using similar techniques (slow cooling in DMSO), Raether and Uphoff (1975) and Simione and Daggett (1976) reported similar results with Entamoeba histolytica and Naegleria bistadialis respectively. Kelly and Campbell (1974) showed that Nippostrongylus brasiliensis larvae survived freezing in liquid nitrogen vapor in normal saline alone; 10% DMSO had no beneficial effect while 10% glycerol had an adverse effect. Furthermore, only exsheathed larvae survived (15-64%) while no motility or infectivity could be demonstrated with frozen non-exsheathed larvae. McCall et al (1975) who froze Dipetalonema vitae larvae at 1° C./min. in 5% DMSO found that infectivity could not be demonstrated with larvae frozen in free suspension even though the larvae were motile after thawing. Conversely, larvae frozen in the intact host (tick) were just as motile and infective after thawing as were unfrozen controls.

Critique - Most of the remarks made in the text of previous sections and critiques after those sections would apply here as well.

Summary - It is clear that the techniques involved in freezing protozoans, in general, were established long ago and have undergone only minor changes. This applies equally well to freezing malarial sporozoites and blood cell stages. The lack of proper control and quantitation in these studies provides sufficient evidence to conclude only that some organisms do indeed survive freezing but the absolute viability and optimal conditions for achieving maximal viability are not known. Finally there appears to be no information available regarding the freezing of gametocytes or their subsequent infectivity in mosquitoes other than Young (1976), who has attempted to freeze gametocytes but without success.

Evaluation of Review - Because of the small number of publications dealing with freezing malarial parasites and the repetitious nature of those in print, I would suggest that a review of the subject for publication is not warranted. Conversely, the inclusion of some of this material in a paper presenting new data would be most appropriate. Therefore, a manuscript is currently in preparation tentatively entitled "Long-Term Cryobiological Storage of Malarial Organisms in the Blood and Exo Erythrocytic Stages." This study, initiated by Strome and Beaudoin in 1967, was designed to determine the effects of long-term storage (10 years) on the infectivity of P. fallax in turkeys. Aliquots have been tested periodically since 1967 and a 10 year specimen is due to be evaluated in the immediate future.

Bibliography

Bafort, J. (1968). The effects of low temperature preservation on the viability of the sporozoites of Plasmodium berghei. Ann. Trop. Med. and Parasit. 62:301.

Booden, T. and Gieman, Q. M. (1973). Plasmodium falciparum and P. knowlesi; Low temperature preservation using Dimethylsulfoxide. Exp. Parasit. 33:495-498.

Dalgliesh, R. J. and Mellors, L. T. (1974). Survival of the parasitic protozoan, Babesia bigemina, in blood cooled at widely different rates to -196° C. Int. J. Parasit. 4:169-172.

Diamond, L. S. (1964). Freeze-preservation of Protozoa. Cryobiol. I (2):95-102.

Diffley, P., et al (1976). An improved method of cryopreservation of Trypanosoma (nannomonas) congolense broden in liquid nitrogen. J. Parasit. 62 (1):136-137.

Diggs, C., et al (1975). Protein synthesis in vitro by cryopreserved Plasmodium falciparum. Amer. J. Trop. Med. and Hyg. 24 (5):760-763.

Filardi, L. S. and Brener, Z. (1975). Cryopreservation of Trypanosoma cruzi bloodstream forms. J. Protozool. 21 (2): 398-401.

Jakstys, B. P., et al (1974). Ultrastructural analysis of Plasmodium berghei sporozoite antigens prepared by freeze thawing and heat inactivation. J. Protozool. 21(2):344-348.

Jeffery, G. M. (1957). Extended low temperature preservation of human malarial parasites. J. Parasit. 43:448.

Jeffery, G. M. (1962). Survival of trophozoites of Plasmodium berghei and Plasmodium gallinaceum in glycerolized whole blood at low temperatures. J. Parasit. 48 (4):601-606.

Jeffery, G. M. and Rendtorff, R. C. (1955). Preservation of viable human malaria sporozoites by low temperature freezing. Exp. Parasit. 4:445-454.

Kelly, J. D. and Campbell, W. C. (1974). Survival of Nippostrongylus brasiliensis larvae after freezing over liquid nitrogen. Int. J. Parasit. 4:173-176.

Kocan, R. M. et al (1967). Freeze preservation of Leucocytozoon simondi sporozoites in liquid nitrogen. J. Protozool. 14(4):726-731.

Lumsden, W. H. R. (1972). Principles of viable preservation of parasitic protozoa. J. Parasit. 2:327-332.

McCall, J. W., et al (1975). Cryopreservation of infective larvae of Dipetalonema vitae. J. Parasit. 61(2):340-342.

Neal, R. A., et al (1974). Survival of Entamoeba and related amoebae at low temperature - II. Viability of amoebae and cysts stored in liquid nitrogen. Int. J. Parasit. 4:353-360.

Raether, W. and Uphoff, M. (1975) Survival of monoxenically (Crithidia sp.) and axenically grown Entamoeba histolytica cultures after storage in liquid nitrogen. Int. J. Parasit. 6:121-125.

Schneider, N. D. and Nella Seal. (1973). Influence of various cooling and warming temperatures on survival after thawing of cryopreserved Plasmodium berghei. Cryobiol. 10:67-77.

Schneider, N. D. Shefner, A. M. (1970). Extended low temperature preservation of a highly lethal Plasmodium berghei strain. Am. J. Vet. Res. 31 (1):173-179.

Simione, F. P. and Daggett, P. M. (1976). Freeze preservation of pathogenic and non pathogenic Naegleria species. J. Parasit. 62:49.

Strome, C. P. A. (1976) Unpublished results.

Walker, P. J. and Ashwood-Smith, M. J. (1960). Dimethyl sulphoxide an alternative to glycerol for the low temperature preservation of trypanosomes. An. Trop. Med. and Parasit. 55:93-96.

Weathersby, A. B. (1976). Freeze dried Plasmodium gallinaceum. (Abst) 50th Annual Mtg. Amer. Soc. Parasitologists.

Wilson, I. and Farrant, J. (1976). Preservation of Plasmodium knowlesi by a two step cooling procedure. (Abst.) Cryobiol. mtg.

Wolfson, Fruma. (1945). Effect of preservation by freezing upon the virulence of Plasmodium for ducks. Amer. J. Hyg. 42(2):155-166.

Young, M. (1976) Personal Communication.